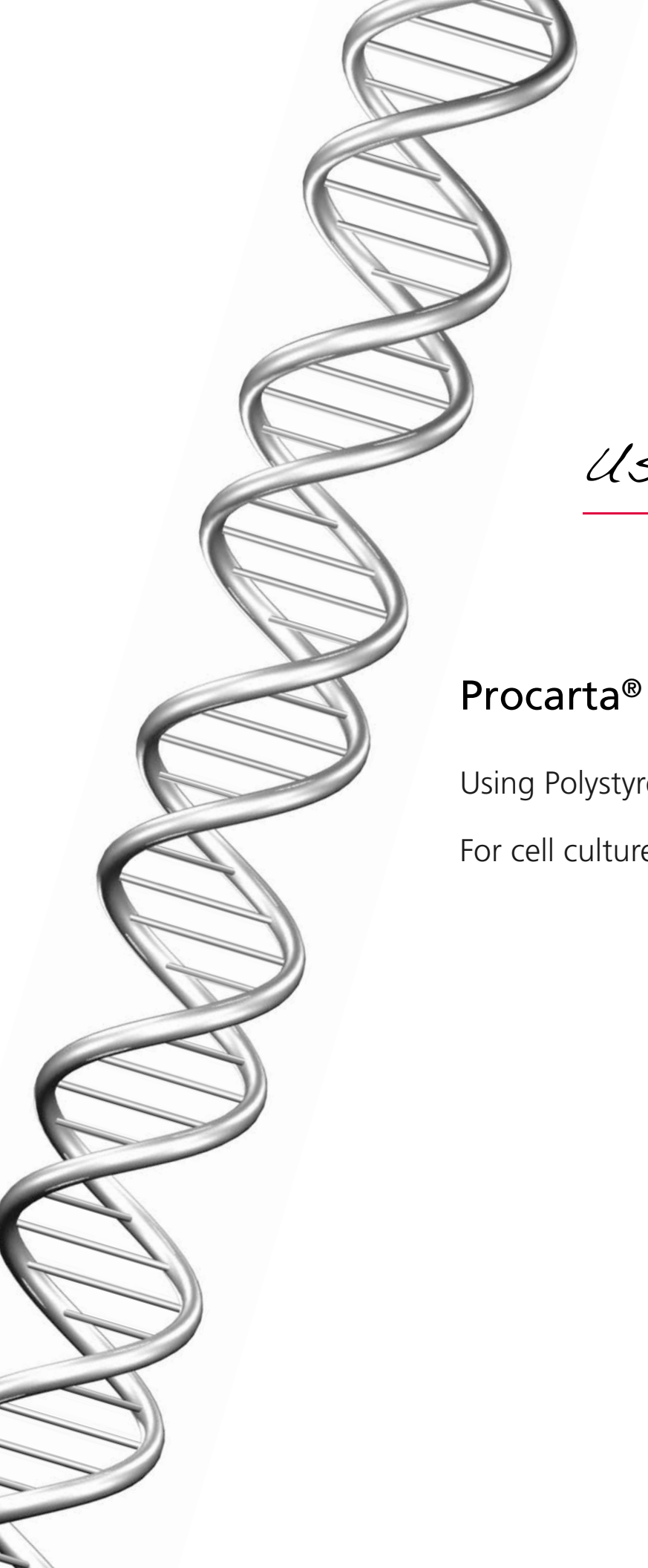

Affymetrix® | Panomics Solutions

User Manual

Procarta® Immunoassays

Using Polystyrene Beads

For cell culture supernatant samples.

P/N 10482-CC Rev.A 020912

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Citing Procarta® Immunoassay in Publications

When describing a procedure for publication using this product, please refer to it as the Procarta® Immunoassay from Affymetrix.

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Intended Use

This user manual is for a Procarta® Immunoassay Kit - Polystyrene Beads from Affymetrix to perform quantitative, multiplexed immunoassays based on the Luminex® technology. The procedure is for simultaneous measurements of multiple protein biomarkers in cell culture supernatant samples. The assay protocol and reagents supplied are not compatible with other manufacturer's reagents. Each 96-well plate kit is configured to allow for the following usage: 16 wells for an 8-point standard curve (in duplicate), 2 wells for blanks, and up to 78 wells for samples. Procarta Immunoassay kits can be stored for up to 6 months from the date of receipt when stored at recommended temperatures.

NOTE: For the most current version of user documentation, go to our website at www.panomics.com

Contacting Technical Support

For technical support, contact the appropriate resource provided below based on your geographical location. For an updated list of FAQs and product support literature, visit our website at www.panomics.com.

Location	Contact Information
North America	1-877-726-6642 option 1, then option 3; pqbhelp@affymetrix.com
Europe	+44 1628-552550 techsupport_europe@affymetrix.com
Asia	+81 3 6430 430 techsupport_asia@affymetrix.com

About Procarta Immunoassay Kits

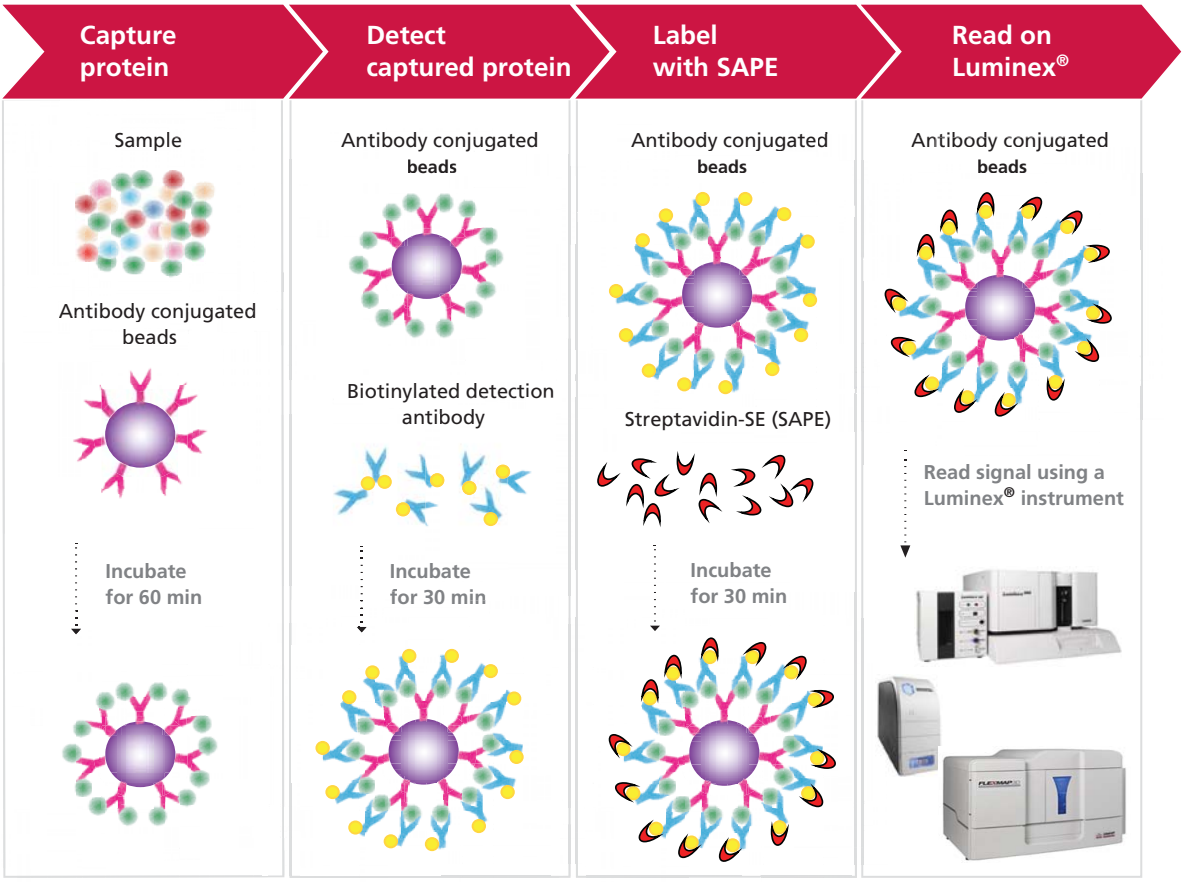
Procarta Immunoassay Kits are available as:

- Standard pre-mixed panels
- “By Request” user configured panels
- New custom assay development for analytes not listed on our website

Procarta Immunoassay Kits contain all the reagents required to run the assays. Please order an appropriate standard diluent buffer for a specific matrix. Please contact your local Affymetrix sales representative for new custom assay development for analytes not listed on our website www.panomics.com.

How it Works

Procarta® Immunoassays use the xMAP® technology (multi-analyte profiling beads) to enable the detection and quantitation of multiple protein targets simultaneously in diverse matrices. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), dual laser design and digital signal processing to effectively allow multiplexing of up to 100 unique assays within a single sample. The Procarta Immunoassay kits are compatible with all Luminex and Luminex-based instruments currently available.



Procarta Immunoassay Kit Contents and Storage Conditions

The Procarta Immunoassay Kit contains the following components listed below. The kits are available in single 96-well plate or ten 96-well plate formats. Refer to the Package Insert for quantities and details of components supplied. The kits are shipped with blue ice. Shelf-life of the kit is 6 months from date of receipt when stored at 2-8 °C. The kits are also supplied with the following inserts:

- **Packaging Insert**: Describes the products included in the kit.
- **Premixed Standard Insert**: Lists the lot number and starting antigen values used standard value calculation.
- **Bead Analyte Association Insert**: Lists the bead number and analyte number.

Component	Description
Antigen Standards, premixed, lyophilized (2 vials each lot for a 1 plate kit)	Recombinant proteins in lyophilized powder. Do not reuse, discard after use. Please note that more than 1 lot of vials may be shipped with each kit. Review the Premixed Standard Insert prior to use.
Detection Antibody, premixed	Detection antibodies in aqueous buffered solution
Antibody Polystyrene Beads	Capture antibodies conjugated to microspheres in aqueous buffered solution. The Bead ID's are printed on the inside flap of the Kit Box and also listed in the Bead Analyte Associate Insert.
Streptavidin-PE (SAPE)[1]	Streptavidin-conjugated R-phycoerythrin in aqueous buffered solution
10X Wash Buffer[1]	Concentrated aqueous buffered solution
Reading Buffer[1]	Aqueous buffered solution
PCR 8-Tube Strip	0.2 mL polypropylene PCR 8-tube strip
Filter Plate and Lid	96-well, Filter plate used with the vacuum manifold
Plate Seals	Adhesive-backed foil plate sealer

[1] *Contains sodium azide. See WARNING below.*

WARNING: All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and be used according to the principles of good laboratory practice.

WARNING: This kit contains small quantities of sodium azide. Sodium azide is highly toxic and reactive in the pure form. At this product's concentration, though not classified as hazardous, build up of sodium azide may react with lead and copper plumbing to form highly reactive explosive metal azide. Dispose of the product in accordance with all State and local regulations.

Required Equipment and Materials Not Supplied

Required Equipment/Material	Source	Part Number
Vacuum Manifold	Millipore	
Microplate shaker	Labline	4625 or equivalent (must have 3 mm orbit with ability to maintain 500 rpm)
Luminex or Luminex-based instrument	MiraiBio, Bio-Rad or other Luminex instrument providers	
Vortex mixer	Major laboratory supplier (MLS)	
Adjustable single and multi channel precision pipettes for dispensing 1-20 µL, 20-200 µL and 200-1000 µL	MLS	
Reagent reservoirs, 25 mL and 100 mL capacity	Vist Labs Corning Costar	3054-1002 or equivalent CLS 4873 or equivalent
Double-distilled (dd) water (H_2O)	MLS	
Microcentrifuge	MLS	

Precautions and Technical Hints

- Thoroughly read this user manual and product insert that is included with the assay kit. The product insert may contain specific instructions for proper use of the “By Request” or custom panels.
- Before starting the assay, turn on the Luminex machine and initiate the startup protocol. It takes 30 min for the lasers to warm-up. Make sure the Luminex machine is calibrated according to the manufacturer’s instructions.
- Some samples may contain high analyte concentrations and require sample dilution for accurate quantitation. Use the cell culture media used to prepare the cells.
- When working with samples and standards, change the pipette tips after every transfer and avoid creating bubbles when pipetting.
- During the incubation steps, cover the 96-well plate with aluminum foil to minimize exposure of the beads to light.
- Be careful not to invert the Filter Plate during the assay or allow contents from one well to mix with another well.
- Use a multi-channel pipette and reagent reservoirs whenever possible to achieve optimal assay precision.
- Store the detection antibody, antibody beads, Streptavidin-PE, samples, and reconstituted standards on ice before adding to the Filter Plate.
- For frozen samples, thaw completely on ice and mix well prior to running the assay.
- Seal all unused wells with an enclosed Plate Seal to ensure proper vacuum pressure.
- When sealing the Filter plate with a Plate Seal, gently press your finger over the Plate Seal. Too much pressure can force the fluid through the filter plate. To avoid Filter Plate leakages, do not seal Filter Plates using a rubber roller (or equivalent) as they apply significant pressure resulting in leakage.

Sample Preparation

Cell culture supernatant samples can be obtained from adherent and non-adherent cells. A total volume of 50 µL/well of cell culture supernatant is needed and a minimum of 2 replicates is recommended.

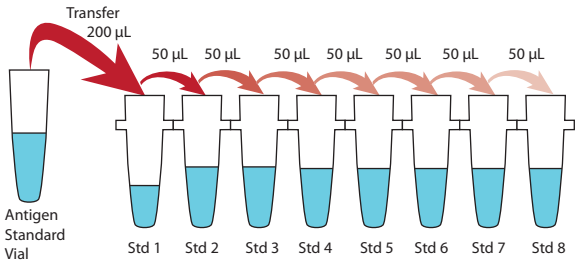
Some samples may contain high concentrations of the analytes. Dilution of the samples may be needed if the analyte concentration is above the assay upper limit of quantitation. Serial dilution of the samples may need to be prepared to determine the appropriate dilution factor for accurately measuring the analytes of interest. Use the same fresh culture medium used for culturing the experimental cells to prepare dilutions of the samples.

If the sample contains cells, centrifuge the sample at 300 x *g* for 5 min at 2-8 °C. Collect the supernatant. Use immediately or aliquot and store at -80 °C.

The TGF-β assay requires a special sample preparation procedure. The TGF- β assay can only detect the active form of TGF- β. The samples must be acid treated and then neutralized to convert the complexed form of TGF- β to its active form. The assay should be processed as a single plex assay since the sample must be acid treated. The TGF-β sample preparation protocol can be found on our website: www.panomics.com.

Preparing Antigen Standards

This section provides instructions on how to make a 4-fold, 8-point standard curve for the assay panel. The antigen standards should be prepared after sample preparation is completed. The serially diluted antigen standards should be added to the assay plate at the same time the samples are added. Each1 plate kit is shipped with two vials of identical antigen standards from the same lot. In some cases an additional set(s) of standards from a different lot may be included in the kit. Please refer to the **Premixed Antigen Standard Insert** when assigning the Standard 1- Standard 8 antigen values for each analyte.

Step	Action
Step 1. Reconstitute Lyophilized Antigen Standards	**A.** Instructions for assay panels with only 1 standard lot in the kit: 1. Centrifuge the antigen standard vial at 2000 x *g* for 10 sec. 2. Add 250 µL of fresh cell culture media into the vial. 3. Vortex gently for 30 sec. 4. Incubate on ice for 5-10 min. **B.** Instructions for assay panels with more than one standard lots in the kit: 1. Centrifuge all the antigen standard vials with different lot numbers at 2000 x *g* for 10 sec. 2. Add 250 µL of fresh cell culture media into one of the vials. 3. Incubate the vial on ice for 5-10 min. 4. Vortex gently for 30 sec. 5. Transfer the entire content into the second vial with a different lot number. 6. Incubate on ice for 5-10 min. 7. Vortex gently for 30 sec. 8. If more than 2 lots of antigen standards are in the kit, repeat steps 5-7 until all the vials with different lot numbers are reconstituted. **NOTE:** **For panels with liquid antigens, follow the instructions on the Package Insert.**
Step 2. Prepare 4-Fold Serial Dilution	**A.** Prepare a 4-fold serial dilution of the reconstituted standard(s) using the PCR 8-tube strip provided. **B.** Add 200 µL of the reconstituted antigen standards into the first tube of the strip tube and label as Standard 1 (Std 1). **C.** Add 150 µL fresh cell culture media into Tubes 2-8. **D.** Using a P-200 pipette, transfer 50 µL of the reconstituted antigen standards from Tube 1 into Tube 2. **E.** Mix by pipetting up and down for a total of 10 times. **F.** After changing the pipette tip, transfer 50 µL of the mixed standards from Tube 2 into Tube 3 **G.** Mix by pipette up and down 10 times. **H.** Repeat Actions D to G for the rest of the tubes to prepare Std 4-8. 

Performing the Assay

Step	Action
Step 1. Prepare 1X wash buffer	Bring the 10X Wash Buffer to room temperature and vortex for 15 seconds. Mix 20 mL of the 10X Wash Buffer with 180 mL ddH20. **NOTE: 1X wash Buffer can be stored at 2-8 °C for up to 6 months. Bring the buffer to room temperature prior to use.**
Step 2. Prepare the Filter Plate	**A.** Mark the standard, sample and blank wells. For your convenience, a blank layout is provided in the Plate Layout section. **B.** Seal the un-used wells of the plate with a Plate Seal prior to starting the assay. Keep the un-used wells of the plate sealed throughout the whole assay procedure. Place the Filter plate on top of the inverted Filter Plate Lid. The plate should be sitting on the hollow end of the lid and not touching any of the surfaces, except for the borders of the lid. This will prevent the bottom of the Filter Plate from against any surface which can lead to leakage. **C.** Pre-wet the Filter Plate by adding 150 µL of Reading Buffer into each well. **D.** Incubate for 5 minutes at room temperature **E.** Remove the Reading Buffer with vacuum filtration. Reading Buffer should clear wells within 8-10 seconds. See "Setting Up and Calibrating the Manifold" at the end of this manual
Step 3. Add the Antibody Polystyrene Beads	**A.** Vortex the premixed Antibody Beads for 30 seconds at room temperature. **B.** Add 50 µL of Antibody Beads to each well. **C.** Remove buffer with vacuum filtration.
Step 4. Wash Antibody Beads	**A.** Add 150 µL of 1X Wash Buffer into each well and remove by vacuum filtration. **B.** Blot the bottom of the Filter Plate thoroughly with paper towels to remove residual buffer.
Step 5. Add Standards and Samples	Add 50 µL of standards or 50 µL of samples as marked on the plate layout sheet into each well. For blanks, add 50 µL of cell culture media.
Step 6. Incubate the Filter Plate	**A.** Gently seal the Filter Plate gently using a Plate Seal provided, place the Filter Plate onto the Filter Plate Lid and completely wrap with aluminum foil. **B.** Room temperature incubation: 1. Shake the 96-Well Plate at 500 rpm for 60 min at room temperature. 2. Proceed to step 7. **C.** Alternatively, the 96-Well Plate can be incubated overnight. 1. Shake the Filter Plate at room temperature for 30 min. 2. Transfer the plate to 4 °C and store on a level surface. 3. After incubation, remove the Filter Plate from 4 °C and shake for 30 min at room temperature. 4. Proceed to step 7.
Step 7. Wash the Filter Plate	**A.** Carefully remove the Plate Seal to avoid splashing the plate contents. **B.** Remove solution with vacuum filtration. **C.** Add 150 µL of 1X Wash Buffer into each well then immediately remove with vacuum filtration. **D.** Wash the plate three times and after the 3rd wash, thoroughly blot the bottom of the Filter Plate with paper towels. **NOTE: When washing the Filter Plate, we recommend using a multi-channel pipette and a large plastic reservoir for the wash buffer or a multi-channel automatic liquid dispenser. Avoid touching the pipette tips to the sides of the wells when adding wash buffer using a multi-channel pipette.**
Step 8. Add Premixed Detection Antibodies	**A.** Add 25 µL of Detection Antibodies into each well. **B.** Gently seal the Filter Plate with a new Plate Seal. **C.** Set the Filter Plate onto the Filter Plate Lid and Wrap with aluminum foil. Shake at 500 rpm for 30 min at room temperature.

Step	Action
Step 9. Wash the Filter Plate	**A.** Repeat Step 7.
Step 10. Add SAPE	**A.** Vortex the SAPE solution vial for 20 sec. **B.** Add 50 µL of SAPE solution into each well. **C.** Gently seal the Filter Plate with a new Plate Seal. **D.** Set the Filter Plate onto the Filter Plate Lid and wrap with aluminum foil. Shake at 500 rpm for 30 min at room temperature.
Step 11. Wash the Filter Plate	**A.** Repeat Step 7.
Step 12. Prepare the Filter Plate for Analysis on a Luminex Instrument	**A.** Add 120 µL of the Reading Buffer into each well. **B.** Seal the Filter Plate with a new Plate Seal. **C.** Wrap the Filter with aluminum foil and shake at 500 rpm for 5 min at room temperature. **D.** Remove the Plate Seal prior to reading on the Luminex instrument. **NOTE: We recommend reading the assayed samples on the Luminex instrument immediately. However, the Filter Plate can be wrapped with aluminum foil and stored for up to 48 hours at 4 °C before proceeding. After storage, shake at 500 rpm for 5 min at room temperature prior to reading. Delay in reading the assayed samples may result in decreased sensitivities for some assays.**

Setup of the Luminex Instrument

Software	Sample Size	DD Gate	Timeout	Bead Event/Bead Region
Luminex 100/ IS100 v 2.318	100 µL	7,500 - 15,000	45 sec	50-100
Bioplex/ Bio-Rad	100 µL	4,335 - 10,000	45 sec	50-100
Miraibio/ Hitachi	100 µL	2,000 - 15,000	45 sec	50-100
Starstation/ ACS	100 µL	4,335 - 10,000	45 sec	50-100

If you are running assays on your Luminex instrument that uses both Plates for Magnetic Beads and Filter Plates for Polystyrene Beads, verify the probe height for each plate type before reading. Failure to adjust the probe height can cause damage to the instrument.

The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for Procarta Immunoassays.

Please refer to the label inside of the Kit Box or the **Bead Analyte Association Insert** for bead region-analyte associations when entering the information into the Luminex acquisition software (xPonent, Bio-Plex, MasterPlex, StarStation).

Please also refer to the **Premixed-Antigen Standard Insert** when assigning the Standard 1 (Std 1) concentration into the analysis software. Each analyte may have a different Std 1 concentration. A 4 fold dilution should be applied to each subsequent standard (Standard 2-8). For example if the starting concentration was 20,000, then a 4 fold dilution for Std 1-8 would be 20,000, 5000. 1250, 312, 78, 19.5, 4.8 and 1.2 pg/ml.

NOTE: If there is a malfunction of the Luminex instrument or software during the run, the Filter can be re-read. Remove the Filter Plate from the instrument and vacuum filter the plate. Resuspend the beads in 120 µL of Reading Buffer, seal with a new Plate Seal and shake at 500 rpm for 5 min at room temperature. The assayed samples may take longer to read since there will be less beads in the wells.

Analyzing Results

The concentration of the samples can be calculated by plotting the expected concentration of the standards against the MFI generated by each standard. A 4PL or 5PL algorithm is recommended for the best curve fit.

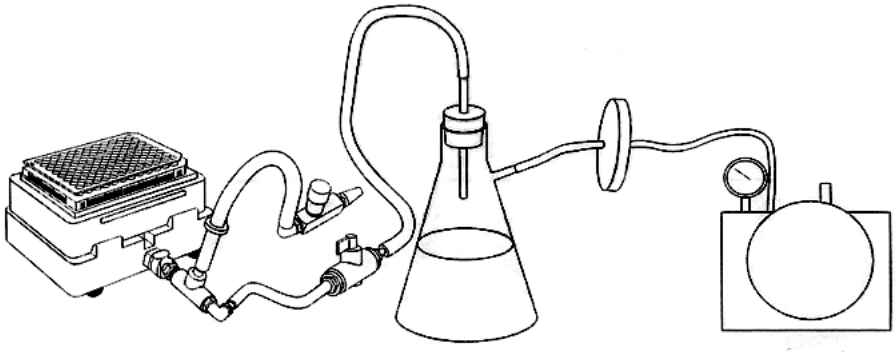
Analyze the assayed samples according to the operation manual for the Luminex or Luminex-based instrument.

Troubleshooting

Observation	Probable Cause	Recommend Solution
Low Flow Rate	Partial Blockage of the flow cell	Remove the Filter plate and perform a wash and rinse cycle.
	Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
High CVs	Samples and antigen standards not stored on ice	Prepare the samples and standards on ice before setting up the assay.
	Contamination from re-using the Plate Seal	Use a new Plate Seal for each incubation step.
	Contamination from contents from adjacent wells	Avoid splashing the Wash Buffer during wash steps into adjacent wells
	Poor pipetting techniques	Use appropriate pipetting techniques. Use new pipette tips for each well during sample and standard addition. Avoid touching pipette tips to sides of the wells when adding wash buffer.

Observation	Probable Cause	Recommend Solution
Low bead count	Probe height is incorrect	Refer to the Luminex manual for proper adjustment of the needle height.
	Reading buffer volume added in the last step to resuspend the beads is too low	Add 120 µL Reading Buffer into each well and shake at 500 rpm for 5 min at room temperature to resuspend the beads prior to reading on the Luminex instrument. Make sure sample size is set at 100 µL in the acquisition protocol.
	High bead aggregation	Vortex the bead suspension well before using in the assay and ensure that the beads are properly mixed during the incubation steps.
	Dyes contained in the beads are photo-bleached from overexposure to light	Store bead solution in the dark and protect the Filter Plate from light by wrapping the Filter Plate with aluminum foil.
	Partial blockage of the flow cell	Remove the Filter Plate and perform a wash and rinse to the instrument
	Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations
	Beads settle on the bottom of the well	Confirm that the plate shaker is set to 500 rpm and shaking for at least 5 min before reading.
	Air bubble in the sample loop	Refer to the Luminex manual for proper removal of the air bubble.
Low signal or sensitivity	Standards not reconstituted and diluted correctly	Prepare fresh antigen standards following the instructions in the Preparing Antigen Standards Section.
	Expired reagents were used	Reagents are good for 6 months from the date of receipt. Do not use expired reagents.
	Suboptimal assay conditions	Follow the recommended incubation times and temperature. Shake the Filter Plate during all incubations except during optional overnight incubation step (Step 7C).
Poor accuracy	Did not use the appropriate assay diluents	Use the same sample type-specific standard and assay buffers for standard and sample preparations.
	Samples and antigen standards were not stored on ice	Prepare and store the samples and standards on ice before setting up the assays

Setting up and Calibrating the Vacuum Manifold

Step	Action
Step 1. Set Up Vacuum Manifold	
Step 2. Calibrate Pressure	**A.** Place the Filter Plate on top of the manifold and Turn on the vacuum. **B.** Press down on all 4 corners of the Filter Plate to form a tight seal. **C.** Adjust the pressure so that it takes 4-6 seconds to evacuate 150 µL of wash buffer from the wells. If the vacuum is too high, beads can get trapped or pulled through the filter. **D.** Turn off vacuum as soon as the solution filters through the wells and remove the plate from the manifold.
Step 3. Operating the Manifold	For all filtration steps, turn on the vacuum pump, place the Filter Plate onto the vacuum manifold and then filter the solution. Avoid splashing and cross-contamination of wells during all wash steps. **NOTE: Do not allow the Filter Plates to air-dry following washes and immediately add the next component after each filtration step. We recommend performing all the wash steps next to the manifold to minimize the amount of time that the beads are exposed to air.s.**
Step 4.	Following the last wash in each series, blot the bottom of the Filter Plate thoroughly with a paper towel to remove traces of 1X Wash Buffer. Avoid touching the bottom of the Filter Plate with your fingers or to the bench during manipulations. Immediately move to the next step to ensure that the beads are in the appropriate buffered solution.

Example Plate Layout

Standards		Samples									
Standard 1	Standard 1	1	1	8	8	16	16	24	24	32	32
Standard 2	Standard 2	2	2	9	9	17	17	25	25	33	33
Standard 3	Standard 3	3	3	10	10	18	18	26	26	34	34
Standard 4	Standard 4	4	4	11	11	19	19	27	27	35	35
Standard 5	Standard 5	5	5	12	12	20	20	28	28	36	36
Standard 6	Standard 6	6	6	13	13	21	21	29	29	37	37
Standard 7	Standard 7	7	7	14	14	22	22	30	30	38	38
Standard 8	Standard 8	Blank	Blank	15	15	23	23	31	31	39	39

Blank Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												